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## Synthesis and Binding Behaviour of New Isomers of Bis-Thiourea (Perilaku Sintesis dan Pengikatan Isomer-Isomer Baharu Bis-Tiourea)

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### ABSTRACT

The two new symmetrical bis-thiourea compounds, 2,2'-[{(terephthaloylbis(azanediyl)bis(carbonothioyl)bis(azanediyl))bis(4-methylpentanoic acid)] **1A** and 2,2'-[{(isophthaloylbis(azanediyl)bis(carbonothioyl)bis(azanediyl))bis(4-methylpentanoic acid)] **1B** have been synthesized by reacting terephthaloyl/isophthaloyl chloride and L-leucine in high yields. Newly synthesized bis-thiourea derivatives were characterized using FTIR, 1D NMR, 2D NMR, MS spectrometry and Elemental analysis. Their binding properties with various cations were also carried out using UV-vis titration experiments. Both isomers exhibited effective binding against  $\text{Ag}^+$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Fe}^{3+}$  in the presence of other cations, such as  $\text{Na}^+$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sn}^{2+}$ , and  $\text{Al}^{3+}$ . Stoichiometries of the host-guest complexation for both isomers were found to be 1:4 by plotting molar-ratio curves. Pearson Product moment correlation coefficient was in the range of 0.83-0.99 and nonlinear regression equation was used to calculate dissociation constant ( $K_d$ ). Both compounds displayed weak antibacterial activities against gram-positive and gram negative bacteria. Cytotoxicity testing on CCD841 Normal human colon epithelial cell line showed that both compounds are non-toxic, with an  $\text{IC}_{50}$  value of 1.50 mg/mL.

**Keywords:** Bis-thiourea; binding studies; host-guest complexation; L-leucine

### ABSTRAK

Dua sebatian baru bis-tiourea bersimetri, 2,2'-[{(tereptaloilbis(azanedil)bis(karbonotiol)bis(azanedil))bis(4-metilpentanoik asid)] **1A** dan 2,2'-[{(isoptaloilbis(azanedil)bis(karbonotiol)bis(azanedil))bis(4-metilpentanoik asid)] **1B** telah disintesis melalui tindak balas antara tereptaloil/isoptaloil klorida dan L-leusin dengan peratusan hasil yang tinggi. Terbitan baru bis-tiourea ini dicirikan menggunakan FTIR, 1D NMR, 2D NMR, spektrometri SJ dan analisis jisim; unsur. Kedua-dua isomer menunjukkan pengikatan yang berkesan terhadap kation  $\text{Ag}^+$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{2+}$  dan  $\text{Fe}^{3+}$  dengan kehadiran kation  $\text{Na}^+$ ,  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Sn}^{2+}$  dan  $\text{Al}^{3+}$ . Kompleks perumah-tetamu bagi kedua-dua isomer menunjukkan stoikiometri 1:4 yang dicerap melalui lengkung nisbah molar. Nilai pekali korelasi hasil Pearson berada dalam julat 0.83-0.99 dan persamaan regresi tak linear digunakan untuk mengira pemalar pengikatan ( $K_d$ ). Kedua-dua sebatian ini menunjukkan aktiviti anti-bakteria yang lemah terhadap bakteria gram positif dan gram negatif. Ujian kesitoksikan terhadap sel epitelium manusia, CCD841 menunjukkan bahawa kedua-dua sebatian tersebut tidak toksik, dengan nilai  $\text{IC}_{50}$ , 1.50 mg/mL.

**Kata kunci:** Bis-tiourea; kajian pengikatan; kompleks perumah-tetamu; L-leusin

### INTRODUCTION

Thiourea, an emerging class of compounds, were synthesized for the first time by Nencki (1873). Thiourea is a class of organic compounds primarily composed of carbon, sulfur and hydrogen atoms. Thiourea linkage is the reactive functionality in this group of compounds. All the biological activities of thiourea derivatives are due to thiourea functionality (Imrich et al. 1994).

Thiourea derivatives display an exorbitant range of applications in the field of pharmacy, agro sciences, and analytical chemistry. These derivatives show an extensive range of bioactivities such as anti-viral (Sun et al. 2010; 2006), anti-bacterial (Zhong et al. 2008), anti-fungal (Ke & Xue 2006; Wang et al. 2006), analgetic, herbicidal (Hua et al. 2006; Xiao et al. 2009), plant growth regulating (Ranise et al. 1991), antiaggregant (Ranise et al. 2003), antiarrhythmic drug (Claridge et al. 2008), local anesthetic

(Manjula et al. 2009) and antihyperlipidemic activities (Vig et al. 1998). Some thiourea derivatives are effective antitumor agents and found to inhibit HIV reverse transcriptase (Peng et al. 2011). Bis-thiourea derivatives, on the other hand, have two thiourea functionalities thus showing considerably enhanced activity (Fernandez et al. 2005; Phetsuksiri et al. 2003). It is quite surprising that some bis-thiourea derivatives act as antitumor agent and exhibit cytotoxicity against various cancer cells (Zhang et al. 2001). Aromatic bis-thiourea derivatives also display enhanced antimicrobial, antibacterial (Zulkiplee et al. 2014), antifungal (Fernandez et al. 2005) urease inhibitor (Jamil et al. 2013) properties and their use as epigenetic modulators (Sharma et al. 2010).

Generally, thiourea derivatives are quite stable. The aromatic nuclei along with thiourea moiety are commonly coplanar and give rigidity to the molecule. They also

possess various positions for the substitution of functional groups (Koenig et al. 1979). Thiourea possesses two potential donor atoms (O and S). They show amazingly rich coordination chemistry (Jamil et al. 2013). This remarkable tendency as hosts has facilitated the use of thiourea derivatives in ramifying new applications in the field of binding chemistry (Jumal et al. 2012).

Amino acids and their derivatives are significant constituents of chemical and biological systems. Various methodologies involve the use of metal complexes (Bazzicalupi et al. 2003; Konishi et al. 1994), imprinted polymers (Katz & Davis 1999; Yoshikawa et al. 1999), natural and modified cyclodextrins (Rekharsky et al. 2001; Yakovenko et al. 2007), synthetic macromolecules as calixrenes (Gasparrini et al. 2002; Qing et al. 2007) and various types of acyclic compounds (Breccia et al. 2003; Hayashida et al. 2002; Higashi et al. 2002) as host molecules (Qing et al. 2009) and also bis-thiourea derivatives of amino acids were found to have anti-HIV properties (Al-Masoudi et al. 2010).

Thiourea derivatives are well known to be good ligands for metal ions as they contain S and N atoms which are donor sites available to coordinate with transition metals such as  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ag}^+$ , and  $\text{Cu}^{2+}$  (Carcu et al. 2000; Estevez et al. 2007; Koch 2001), but also present specific effects caused by the conformational isomerism, steric hindrance, presence of donor sites in the substituent groups or existence of intra-molecular interactions. This versatility as ligands has fostered the use of thiourea derivatives towards new applications in the field of chemosensors.

When examining the scientific literature, a number of contributions appear about this topic (Otao et al. 2001) described 46 thiourea derivatives as potential ionophores for ion selective electrodes (ISEs). Some of these were used in potentiometric sensors for heavy metals Pb (II), Cd(II) and Hg(II). The use of thiourea derivatives was also proposed in the voltammetric determination of Pd(II) and Cd(II) (Estevez et al. 2007; Lubert et al. 2002). Another contribution of Singh et al. (2007) described a polymeric membrane electrode with a thiourea derivative as ionophore in the determination of ytterbium with a very low detection limit ( $5.5 \times 10^{-8}$  M).

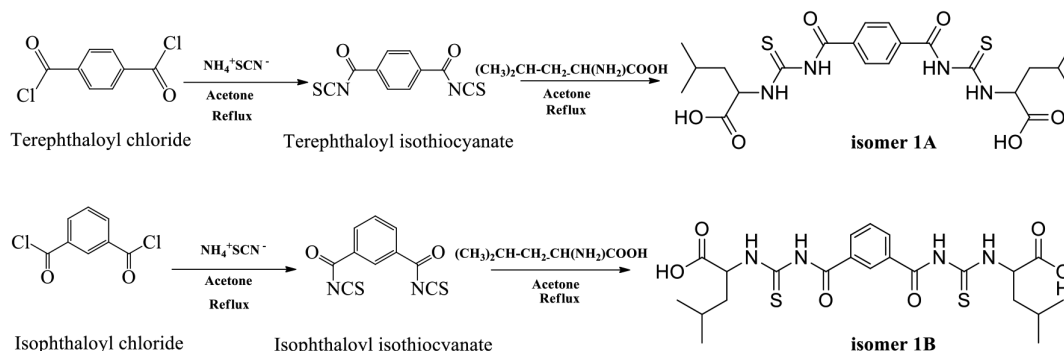
Thiourea derivatives have been generally used in cation selective electrodes by their well-known property of forming complex with metal ions. Opposite to this, there are some reports where the thiourea derivatives are used as neutral carriers in selective electrodes for anions (Nishizawa et al. 1998; Xiao et al. 1997). In the past two decades, only a few thiourea derivatives were studied to be used as metal binding acceptors for heavy metal ions. A Pb(II) ion selective electrode (ISE) was reported and Cd(II). Although several arylthioureas have also been tested as new organic ionophores for heavy metal ISE (Otao-Sanchez et al. 2001), investigations on mercury(II) sensors are still very limited. 1,3-diphenylthiourea as potential ionophores for mercury(II)-ISE was reported by Pérez-Marín et al. (2002). The lack of efficient mercury(II) electrodes certainly has driven the interest to investigate new thiourea ionophores in the preparation of mercury(II) sensors.

In our case, the possibility that bis-thioureas may be forming complexes with heavy metals is an alternative which has not been sufficiently explored but constitutes an interesting option to obtain ISEs for heavy metals with good performance. In the current study, two symmetrical bis-thiourea derivatives (**1A** and **1B**) were synthesized from terephthaloyl and isophthaloyl functionalities, which were used as a spacer and held the central position of the molecule, while, L-leucine was incorporated as a side chain linker (Scheme 1). The newly synthesized derivatives were characterized by FTIR,  $^1\text{H}$ NMR,  $^{13}\text{C}$ NMR, 2DNMR (COSY, NOESY and HMQC), ESI-MS and CHNS Elemental analysis techniques. The binding behaviour of the bis-thiourea was then studied using UV-vis titration experiments against various metal cations. Fourteen strains of bacteria were selected to study the antibacterial activities and CCD841 Normal human colon epithelial cell lines was used for cytotoxicity studies.

## EXPERIMENTAL DETAILS

### MATERIALS AND MEASUREMENTS

All the chemicals utilized were procured from Acros Organics (Geel, Belgium) and Sigma-Aldrich (Saint



SCHEME 1. Synthesis of Bis-thiourea-leucine based isomers 1A and 1B

Louis, MO, USA) and were used as it is. Before using, all solvents were distilled. The melting points were determined by the open tube capillary method using EZ-Melt 78349 instrument (SRS, USA) and are uncorrected. The infrared spectrum (IR) of the product (KBr pellets) was recorded using a Perkin Elmer Spectrum GX spectrophotometer (Perkin Elmer, Waltham, MA, USA) in the range of 400–4000  $\text{cm}^{-1}$  with resolution 4  $\text{cm}^{-1}$ . UV-vis measurements were performed on double beam Varian UV 3.0 (Cary 50, Varian Australia Pty. Ltd.) spectrophotometer with a quartz cuvette (path length: 1 cm) in the range of 200–800 nm with the highest resolution of 1 nm. Nuclear Magnetic Resonance experiments ( $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra) were performed on a Bruker 400 MHz instrument using  $\text{DMSO}-d_6$  as solvent. Electro Spray Ionization-MS spectra were attained on a Micro ToFQ (Bruker, AXS Incorporation, and Madison, WI, United States of America).

#### GENERAL PROCEDURE FOR THE SYNTHESIS OF ISOMERS (1A AND 1B)

A solution of terephthaloyl/isophthaloyl chloride (0.003 mol, 0.609 g), in dried acetone (20 mL) was prepared. Ammonium thiocyanate (0.006 mol, 0.456 g), previously dried at  $80^\circ\text{C}$  for two h, dissolved in dried acetone (15 mL), was added dropwise to the above solution and stirred for 1 h under reflux condition to obtain terephthaloyl or isophthaloyl isothiocyanate. The precipitate of ammonium thiocyanate was filtered off. To the filtrate, a solution of L-leucine (0.006 mol, 0.787 g), in dry acetone (15 mL) was added and stirred for 24–30 h under reflux to get the target substituted bis-thiourea isomers **1A** and **1B** in 91% and 88% yields, respectively (scheme 1). Sufficient ice was then poured into the mixture and the product was collected as a pale yellowish precipitate, which was filtered, washed several times with plenty of water and dried in vacuum desiccator. Recrystallization from Ethanol/DMSO was performed to further purify the product.

#### 2,2'-[{(TEREPHTHALOYL)BIS(AZANEDIYL) BIS(CARBONOTHIOYL) BIS(AZANEDIYL)} BIS(4-METHYLPENTANOIC ACID)] (1A)

(1.398 g, 91%) as a yellowish solid, mp:  $189.5\text{--}191.2^\circ\text{C}$ , [Found: C, 51.87; H, 5.73; N, 10.77; S, 12.47;  $\text{M}^+$ , 533.102.  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_6\text{S}_2$  requires C, 51.75; H, 5.92; N, 10.97; S, 12.56%];  $\nu_{\text{max}}$  (KBr/ $\text{cm}^{-1}$ ) 3429 (O-H), 3246 (N-H), 3051 ( $\text{C}-\text{H}_{\text{arom}}$ ), 2959 ( $\text{C}-\text{H}_{\text{aliph}}$ ), 1731 (C=O), 1658 (COOH), 1550 (C-N), 1529 (Ar-C), 1017 (C=S);  $\delta\text{H}$  (400 MHz,  $\text{DMSO}-d_6$ ,  $(\text{CH}_3)_4\text{Si}$ ) 0.91 (6H, d,  $J=6.4\text{Hz}$ ,  $2\times\text{CH}_3$ ), 0.95 (6H, d,  $J=6.4\text{Hz}$ ,  $2\times\text{CH}_3$ ), 1.69 (2H, m,  $2\times\text{CH}$ ), 1.81 (4H, m,  $2\times\text{CH}_2$ ), 4.93 (2H, q,  $J=2.4\text{Hz}$ ,  $J=7.2\text{Hz}$ ,  $2\times\text{CH}$ ), 8.02 (4H, s, Ar-H), 11.13 (2H, d,  $J=7.6\text{Hz}$ ,  $2\times\text{NH}$ ), 11.76 (2H, s,  $2\times\text{NH}$ ).  $\delta\text{C}$  (150 MHz,  $\text{DMSO}-d_6$ ) 23.0 ( $\text{CH}_3$ ), 22.4 ( $\text{CH}_3$ ), 25.1 (CH), 39.5 ( $\text{CH}_2$ ), 56.6 (CH), 129.0 ( $\text{CH}_{\text{arom}}$ ), 136.3 ( $\text{C}_{\text{arom}}$ ), 168.2 (C=O), 173.0 (COOH), 180.8 (C=S); MS (EI): ( $m/z$ ) = 533. 102 [ $\text{M}+\text{Na}$ ] $^+$ .

#### 2,2'-[{(ISOPHTHALOYL)BIS(AZANEDIYL) BIS(CARBONOTHIOYL) BIS(AZANEDIYL)} BIS(4-METHYLPENTANOIC ACID)] (1B)

(1.346 g, 88%) as a pale yellowish solid, mp.:  $117\text{--}119^\circ\text{C}$ ; [Found: C, 51.65; H, 5.79; N, 10.88; S, 12.47;  $\text{M}^+$ , 533.401.  $\text{C}_{22}\text{H}_{30}\text{N}_4\text{O}_6\text{S}_2$  requires C, 51.75; H, 5.92; N, 10.97; S, 12.56%];  $\nu_{\text{max}}$  (KBr/ $\text{cm}^{-1}$ ) 3246 (N-H), 3067 ( $\text{C}-\text{H}_{\text{arom}}$ ), 2959 ( $\text{C}-\text{H}_{\text{aliph}}$ ), 1724 (C=O), 1676 (COOH), 1547 (C-N), 1514 (Ar-C), 1019 (C=S);  $\delta\text{H}$  (400 MHz,  $\text{DMSO}-d_6$ ,  $(\text{CH}_3)_4\text{Si}$ ) 0.92 (6H, d,  $J=6.0\text{Hz}$ ,  $2\times\text{CH}_3$ ), 0.95 (6H, d,  $J=6.0\text{Hz}$ ,  $2\times\text{CH}_3$ ), 1.70 (2H, m,  $2\times\text{CH}$ ), 1.77 (4H, m,  $2\times\text{CH}_2$ ), 4.93 (2H, q,  $J=2.4\text{Hz}$ ,  $J=7.2\text{Hz}$ ,  $2\times\text{CH}$ ), 7.69 (1H, t,  $J=7.6\text{Hz}$ , Ar-H), 8.13 (2H, dd,  $J_{\text{ortho}}=7.6\text{Hz}$ ,  $J_{\text{meta}}=2.4\text{Hz}$ , Ar-H), 8.53 (1H, s, Ar-H), 11.18 (2H, d,  $J=7.6\text{Hz}$ ,  $2\times\text{NH}$ ), 11.58 (2H, s,  $2\times\text{NH}$ ).  $\delta\text{C}$  (150 MHz,  $\text{DMSO}-d_6$ ) 22.3 ( $\text{CH}_3$ ), 23.0 ( $\text{CH}_3$ ), 25.1 (CH), 39.5 ( $\text{CH}_2$ ), 56.6 (CH), 129.2 ( $\text{CH}_{\text{arom}}$ ), 129.6 ( $\text{CH}_{\text{arom}}$ ), 132.1 ( $\text{CH}_{\text{arom}}$ ), 133.6 ( $\text{C}_{\text{arom}}$ ), 168.0 (C=O), 173.0 (COOH), 180.7 (C=S); MS (EI): ( $m/z$ ) = 533.401 [ $\text{M}+\text{Na}$ ] $^+$ .

## RESULTS AND DISCUSSION

### IR VIBRATIONAL SPECTRA

Bis-thiourea isomers **1A** and **1B** demonstrated nearly same infrared spectra. The O-H stretching frequency for isomer **1A** was found at  $3429\text{ cm}^{-1}$ , whereas for isomer **1B** the O-H signal overlapped with N-H and a relatively broad peak was observed instead. The C-H stretching of aromatic  $\text{sp}^2$  carbon of isomer **1A** and **1B** appeared at  $3051$  and  $3067\text{ cm}^{-1}$ , respectively (Qing et al. 2007). While stretching vibration of  $\text{sp}^3$  alkyl chains appeared at  $2951\text{ cm}^{-1}$  for both isomers **1A** and **1B** (Arslan et al. 2007). The N-H stretching vibration modes for the two isomers appeared at  $3246\text{ cm}^{-1}$ , apparently lower than secondary amines and amides absorption range ( $3300\text{--}3500\text{ cm}^{-1}$ ) owing to the presence of intramolecular H-bond (Tadjarodi et al. 2007) between the hydrogen atom of thio-amide group ( $\text{H}-\text{N}-\text{C}=\text{S}$ ) and the oxygen atom of carbonyl group ( $\text{C}=\text{O}$ ) (Saeed et al. 2014). The lineament frequency of  $\nu(\text{C}=\text{O})$  and  $\nu(\text{C}=\text{O}_{\text{carboxylic}})$  stretching appeared at 1731, 1724 and 1658,  $1676\text{ cm}^{-1}$  for isomer **1A** and **1B**, respectively (Roslan et al. 2009). The  $\nu(\text{C}-\text{N})$  stretching bond appeared at 1550 and  $1547\text{ cm}^{-1}$  for isomer **1A** and **1B**, respectively. The  $\nu(\text{C}=\text{S})$  vibrational stretching frequencies were observed at 1017 and 1019 for isomer **1A** and **1B**, respectively. These lowering vibrational frequencies were due to mesomeric electron releasing nitrogen bonded to thiocarbonyl group as in the case of thioureas. This lowering of  $\text{C}=\text{S}$  stretching frequencies is due to acquiring partial polar character.

### $^1\text{H}$ , $^{13}\text{C}$ NMR, 2D NMR (COSY, NOESY, HMQC) SPECTRA

The isomers were further characterized and confirmed using  $^1\text{H}$ ,  $^{13}\text{C}$  NMR, and 2D NMR spectroscopy. The  $^1\text{H}$  chemical shifts of the amide appeared as a singlet at 11.75, 11.58 ppm, while thioamide proton appeared as a doublet at 11.13, 11.18 ppm for isomer **1A** and **1B**, respectively. The

downfield of amide and thio-amide protons is mainly due to the formation of the intramolecular H-bonding between the amino proton N–H and the oxygen/sulfur atoms of the carbonyl/thiocarbonyl group in addition to the anisotropy effect (Abosadiya et al. 2015). The aromatic protons of isomer **1A** appeared as a singlet at 8.02 ppm, as all the protons are symmetric, whereas the aromatic protons of isomer **1B** show three characteristic signals as of ortho-meta substituted benzene ring at 8.53 ppm as singlet, 8.13 ppm as doublet-of-doublet and 7.69 ppm as a triplet. The chemical shift for chiral –CH– proton observed at 4.93 ppm as a quartet. The downfield signal of the chiral proton is due to the anisotropic effect of carboxylic carbonyl and due to deshielding of electron withdrawing thioamide amine. The resonance signals for diastereotopic –CH<sub>2</sub>– and –CH– protons in both isomers found to overlap thus giving a multiplet. 2D NMR spectroscopy then used to investigate the overlapping of methylene and –CH– protons. The correlation spectroscopy (COSY) spectrum showed the definite interactions of –CH<sub>2</sub>– and –CH– protons. The –CH– proton interacted with the methyl protons, whereas, the methylene protons showed interaction with Chiral –CH– proton. The two different interactions revealed the presence of two different types of protons. The NOESY spectrum showed the spatial interactions of methylene proton and amide –NH– proton. The HMQC spectrum showed a correlation of <sup>1</sup>H and <sup>13</sup>C spectra, thus –CH<sub>2</sub>– and –CH– protons could be seen clearly attached to two different carbons. The –CH<sub>2</sub>– protons resonance signal

found at 1.81, 1.77 ppm and that of –CH– proton was at 1.69, 1.70 ppm, for isomers **1A** and **1B**, respectively. The resonance signals for methyl protons appeared as doublets at 0.95, 0.91 ppm and 0.95, 0.92 ppm for isomers **1A** and **1B**, respectively. Figure 1 show the <sup>1</sup>H NMR, COSY, NOESY and HMQC spectrum of **1A**.

In the <sup>13</sup>C NMR spectra, all the carbons were found in their expected regions (Freeman 1997). The carbon chemical shifts of C=S, C=O<sub>carboxylic</sub> and C=O were found at 180.8, 173.0, 168.2 ppm for isomer **1A** and 180.7, 173.0, 168.0 ppm for isomer **1B**. The aromatic carbon chemical shifts of the isomers appeared in the range of 129.0–136.3 ppm. The chemical shifts of chiral carbon appeared at 56.6 ppm for both isomers. The chemical shift of alkyl groups methylene; –CH– and methyl carbons were observed in the range 23.0–39.5 ppm for both isomers.

## BINDING STUDIES

### UV-VIS SPECTRA MEASUREMENTS

The two isomers **1A** and **1B** were first prepared as a stock solution in DMSO ( $1 \times 10^{-3}$  M). All the metal ions were also prepared as a stock solution in DMSO ( $1 \times 10^{-3}$  M). The work solution was prepared by adding different volumes (0 - 500  $\mu$ L) of metal ion solution into series of measuring flasks, then, the same amount (100  $\mu$ L) of host compound was added to each flask followed by dilution up to the mark by adding DMSO. After shaken

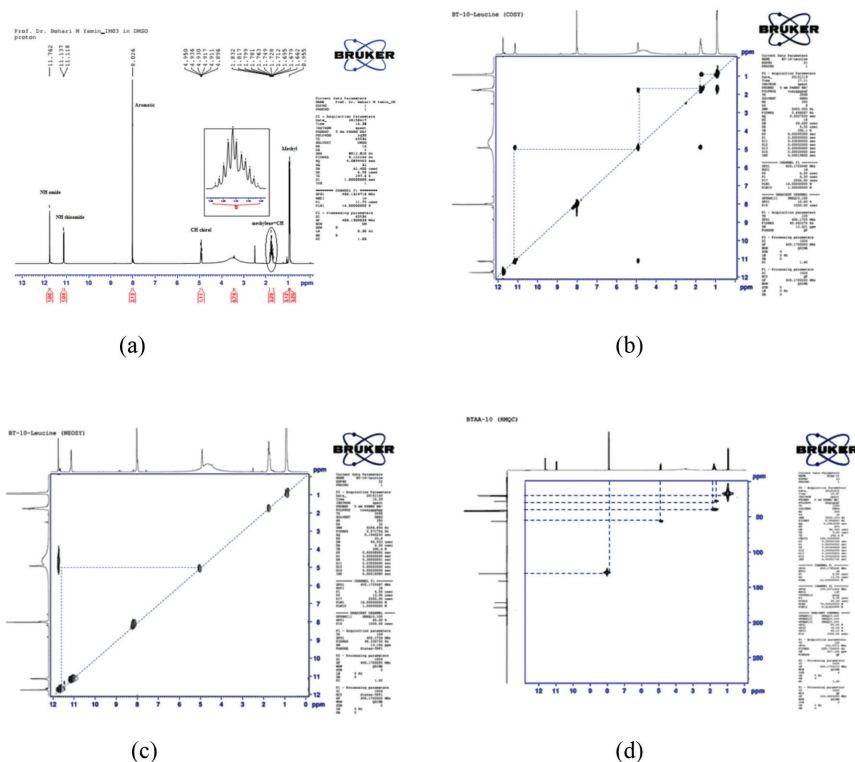


FIGURE 1. (a) <sup>1</sup>H NMR spectrum of **1A** (b) COSY spectrum of **1A** (c) NOESY spectrum of **1A** (d) HMQC spectrum of **1A**



for several minutes, the solution was measured using UV-vis spectrophotometer. Quartz cells (path length 1 cm) were used in the UV-vis range of 200 - 800 nm with the highest resolution of 1 nm. The correlation coefficient was calculated by Pearson product-moment correlation method. The dissociation constants ( $K_d$ ) were calculated by using non-linear regression equation and plotting a fit line curve using sigma plot 12.0 (Systat Software Inc.). The detection limit was calculated by  $3 \sigma/S$ , where ' $\sigma$ ' is the standard deviation and ' $S$ ' is the slope of the fit line in a titration experiment. To show the veracity of data, more than 20 series of continuous data were collected in the UV-vis titration experiments until the absorbance intensities approach equilibrium.

#### THEORY AND CALCULATIONS

The correlation coefficient was used to measure a linear correlation between two variables (Absorbance vs Concentration) during the titration experiments. Pearson product-moment correlation method was used in this study to measure the degree of linear dependence between the two variables. The formula for correlation coefficient ' $r$ ' can be achieved by substituting estimates of the covariance and variance in the formula below.

$$r = r_{xy} = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{n \sum x_i^2 - (\sum x_i)^2} \cdot \sqrt{n \sum y_i^2 - (\sum y_i)^2}}$$

where  $r$  is the correlation coefficient;  $x$  is the concentration;  $y$  is the absorbance; and  $n$  is the no. of observations.

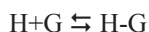
The detection limit was calculated by using the following equation.

$$DL = 3 \sigma / S$$

where  $\sigma$  is the standard deviation of 5 blank readings; and  $S$  is the slope of the fit line in titration experiment.

#### CLARK'S THEORY OF BINDING

Alfred Joseph Clark gave this concept in 1926. For a bimolecular reaction:



Equilibrium dissociation constant ( $K_d$ ) or an equilibrium association constant ( $K_a$ ), which are reciprocally related, as shown below:

$$K_d = \frac{[H][G]}{[H-G]}$$

$$K_a = \frac{[H-G]}{[H][G]}$$

Irrespective of mechanism, all reversible reactions reach an equilibrium distribution of reactants and products when the rates of forward and backward reactions become equal. The overall rate can be expressed as:

$$d[H-G]/dt = k_{\text{assn}}[H][G] - k_{\text{dissn}}[H-G]$$

At the start of reaction association rate ( $k_{\text{assn}}[H][G]$ ) would dominate. As more complexes formed, the association rate would decrease and the dissociation rate would increase. At some point, the rates of the opposing reactions would become equal, which can be expressed as:

$$\begin{aligned} d[H-G]/dt &= -d[H]/dt = -d[G]/dt \\ dt &= k_{\text{assn}}[H][G] - k_{\text{dissn}}[H-G] = 0 \end{aligned}$$

#### UNDER THESE CONDITIONS

This expression shows that the equilibrium concentration of reactants and products will have a constant ratio ( $K_d$ ) that is equal to the ratio of the reverse and forward rate constants.  $K_d$  is called an equilibrium dissociation constant. In the present study binding constant ( $K_d$ ) was calculated by Nonlinear Regression equation using Sigma plot 12.0 (Systat Software Inc.).

For one-site binding, the nonlinear regression equation will be expressed as:

$$y = \frac{B_{\text{max}} \cdot x}{K_d + x}$$

where  $B_{\text{max}}$  is the host-guest complex;  $y$  is the absorbance; and  $x$  is the  $[G]/[H]$ .

For two-site binding, the nonlinear regression equation was modified as:

$$y = \frac{B_{\text{max}_1} \cdot x}{K_{d_1} + x} + \frac{B_{\text{max}_2} \cdot x}{K_{d_2} + x}$$

#### BINDING BEHAVIOUR OF 1A AND 1B

To examine the binding behaviour of isomers **1A** and **1B** against metal ions, titration experiments were carried out. In the absence of metal ions, absorption maxima were observed at 260 and 265 nm, for isomers **1A** and **1B**, respectively, which can be assigned to an intramolecular charge transfer (ICT) absorption band, as the case with thioureas (Qing et al. 2009). Upon gradual addition of metal ions to the solutions, of isomer **1A** and **1B**, only ( $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Ag}^{+}$ ) gave remarkable enhancement of emission intensity at 260 and 265 nm (Figure 2). The increase of emission intensity was attributed to the possible formation of host-guest complex at two probable sites. Amongst the two, the first probable site of complication was carboxylate functionality (Abosadiya et al. 2007), and the second one was thiourea functionality via  $\text{C}=\text{S}$ ,  $\text{C}=\text{O}$  (Li et al. 2014). To investigate the mechanism of binding interactions of isomer **1A** and

**1B** against metal ions, continuous variation titration experiments were carried out. In these experiments, the concentration of the isomers was kept uniform while the concentration of metal ions was gradually increased. Based on these titration experiments, the stoichiometry of complex formation between isomers with metal ions was calculated by plotting a molar-ratio method, the stoichiometry for all metal ions was found to be 1:4 except for 1A-Cu<sup>2+</sup> which was 1:2, which could possibly be because of complexation of Cu<sup>2+</sup> at carboxylic-carbonyl/thionyl group in a twisted conformation of compound 1A (Yanping et al. 1999). Dissociation constant ( $K_d$ ) was calculated by nonlinear regression equation (Table 1) (Khansari et al. 2014). Based on the data of dissociation constant in Table 1, the  $K_{d2}$  values are quite low as compared to the  $K_{d1}$ . This gives an indication of the fact that the high value of equilibrium at carboxylic functionality is the preferred site of complexation over C=S, C=O functionality. By looking at the titration spectra of isomer **1A** and **1B** vs Fe<sup>3+</sup>, Cu<sup>2+</sup>, Hg<sup>2+</sup>, and Ag<sup>+</sup> (Figure 3), the emergence of a new band at 360 - 365 nm, which gradually increased with the incremental addition of metal ions, was due to the effect

of deprotonation of amino proton by the counter anions. A similar phenomenon for a urea-based receptor was also reported by Dey et al. (2010). The absorbance maxima increased linearly with the concentration of all the selected metal ions in a range (0-500  $\mu$ L), the values for correlation coefficient ( $r$ ) and detection limits (DL) based on titration experiments is shown in (Table 1).

#### ANTI-BACTERIAL STUDIES

The anti-bacterial activity of bis-thiourea derivative **1A** and **1B** were examined *in vitro* at concentrations of 50, 25, 12.5, 6.25 and 3.125 mg/mL against marine pathogenic bacterial strains *Lysinibacillus* sp. Gb01 (Gram positive), *Vibrio owensii* Gb04 (Gram negative), *Vibrio alginolyticus* Gb05 (Gram negative), *Loktanella* sp. UKMGb03C (Gram negative), *Vibrio owensii* SS1 (Gram negative), *Vibrio alginolyticus* SS17 (Gram negative) and other pathogenic bacterial strains which includes *Bacillus subtilis* (Gram positive), *Escherichia coli* (Top 10) (Gram negative), *Enterobacter sakazakii* (Gram negative), *Enterobacter shigella* (Gram negative), *Enterococcus faecalis* (Gram

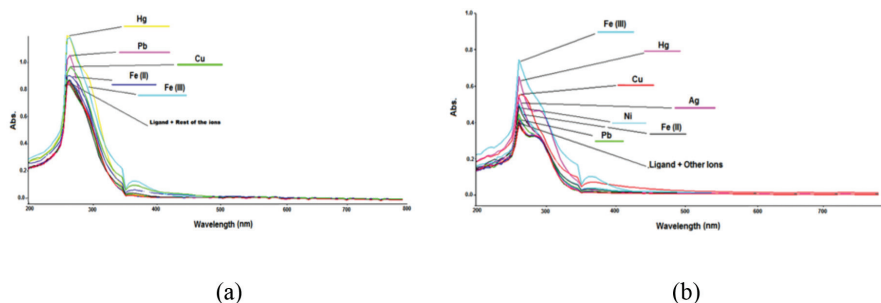


FIGURE 2. (a) Interaction of metal cations with isomer 1A (b) Interaction of metal cations with isomer 1B

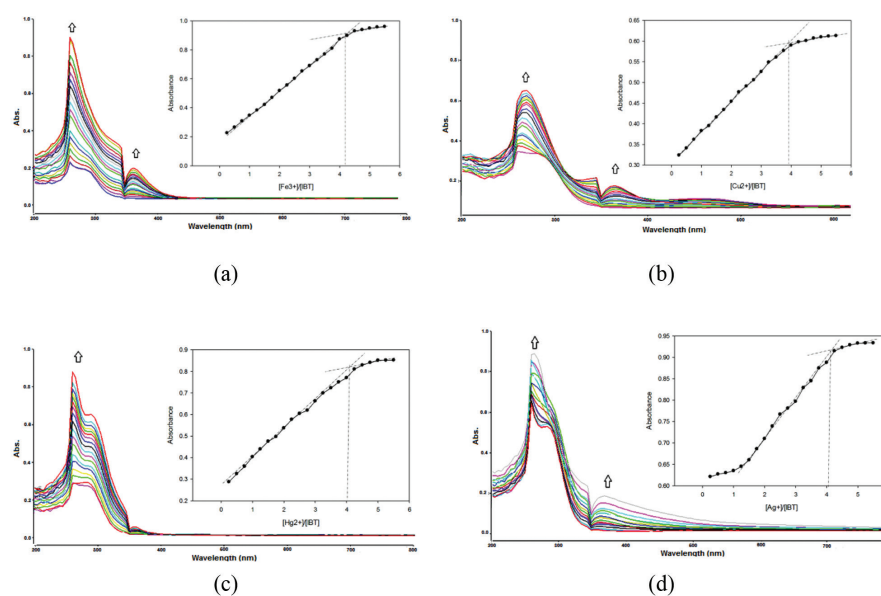


FIGURE 3. Titration data and molar-ratio curve of (a) isomer 1A-Fe<sup>3+</sup> (b) isomer 1A-Cu<sup>2+</sup> (c) isomer 1B-Hg<sup>2+</sup> (d) isomer 1B-Ag<sup>+</sup>

TABLE 1. Binding interaction data of isomer 1A and 1B

Ligand-Cation	r	DL (M)	St-Cplx	Dissociation Constant (M)	
				K <sub>d1</sub>	K <sub>d2</sub>
1A-Fe <sup>3+</sup>	0.872	4.03×10 <sup>-3</sup>	1:4	1.69×10 <sup>1</sup>	4.37×10 <sup>-20</sup>
1A-Fe <sup>2+</sup>	0.985	1.40×10 <sup>-1</sup>	1:4	1.77×10 <sup>5</sup>	2.01×10 <sup>-8</sup>
1A-Cu <sup>2+</sup>	0.833	1.83×10 <sup>-3</sup>	1:2	-	5.07×10 <sup>-3</sup>
1A-Pb <sup>2+</sup>	0.994	3.62×10 <sup>-2</sup>	1:4	2.10×10 <sup>1</sup>	9.45×10 <sup>-18</sup>
1A-Hg <sup>2+</sup>	0.987	1.20×10 <sup>-2</sup>	1:4	6.55×10 <sup>7</sup>	1.35×10 <sup>-17</sup>
1B-Fe <sup>3+</sup>	0.981	3.50×10 <sup>-3</sup>	1:4	5.46×10 <sup>4</sup>	1.90×10 <sup>-25</sup>
1B-Fe <sup>2+</sup>	0.985	4.26×10 <sup>-2</sup>	1:4	9.673	1.34×10 <sup>-18</sup>
1B-Cu <sup>2+</sup>	0.985	3.26×10 <sup>-2</sup>	1:4	7.550	9.55×10 <sup>-18</sup>
1B-Pb <sup>2+</sup>	0.989	2.13×10 <sup>-2</sup>	1:4	7.924	1.17×10 <sup>-17</sup>
1B-Hg <sup>2+</sup>	0.996	1.20×10 <sup>-2</sup>	1:4	1.34×10 <sup>1</sup>	4.93×10 <sup>-19</sup>
1B-Ni <sup>2+</sup>	0.994	3.06×10 <sup>-2</sup>	1:4	1.35×10 <sup>1</sup>	3.48×10 <sup>-18</sup>
1B-Ag <sup>+</sup>	0.991	1.53×10 <sup>-2</sup>	1:4	1.27×10 <sup>7</sup>	1.16×10 <sup>-17</sup>

'r'=correlation coefficient; 'DL'=Detection Limit; 'St-Cplx'= Stoichiometry complexation

positive), *Salmonella typhimurium* (Gram negative), *Escherichia coli* (ATCC) (Gram negative), *Bacillus cereus* (Gram positive) by using agar diffusion technique at 37°C (Larry et al. 1981; Shank & Marmion 1979). The results of bacterial growth inhibition data are given in Table 2.

The various effects of the newly synthesized compound **1A** and **1B** at different concentrations can be expressed by their minimum inhibitory concentration (MIC). The MIC value was determined by extrapolating the concentration at the zero growth rates of different bacteria (Alvarez et al. 2004). MIC values of compounds **1A** and **1B** against different bacteria is shown in (Table 2). The high MIC values against several bacteria shows weak antibacterial activity, hence the data suggest that compounds **1A** and **1B** are unsuitable as a potential antibacterial agent against those bacterial strains (Zirihi et al. 2005).

The presence of C=S, C=O and N-H functional groups in thiourea derivatives are reported to give good

antibacterial activity as they react with carboxyl and phosphate groups of the bacterial surface (Arslan et al. 2009). Derivative **1A**, however, showed no excellent biological activity against various bacteria. It is assumed from the structure of similar bis-thiourea derivative (Fakhar et al. 2017) that the thiourea moiety is not planar because of intermolecular attractions hence offering steric hindrance. The occurrence of steric hindrance creates a force that obstructs the contact between active sites in the compound with receptor site of the bacteria (Fernandez et al. 2005).

#### CYTOTOXICITY STUDY

The cytotoxicity test was performed by using MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Cell culture with the concentration of 2 × 10<sup>3</sup> cells/mL was used into 96-well plates. The diluted

TABLE 2. Antibacterial activities of isomers 1A and 1B

Bacteria	Derivatives 1A						Derivatives 1B					
	Diameter of inhibition zone (mm)					MIC (mg/ml)	Diameter of inhibition zone (mm)					MIC (mg/ml)
	50mg	25mg	12.5mg	6.25mg	3.125mg		50mg	25mg	12.5mg	6.25mg	3.125mg	
<i>L. species</i>	10	9	8	6	6	3.01	16	14	13	10	7	1.495
<i>V. owensii</i>	8	7	6	6	6	12.25	8	7	6	6	6	11.376
<i>V. alginolyticus</i>	7	6	6	6	6	>25	8	8	7	6	6	10.62
<i>L. species</i>	8	7	6	6	6	12.372	15	13	12	9	7	1.248
<i>V. owensii</i>	8	7	6	6	6	12.372	13	12	10	8	7	0.744
<i>V. alginolyticus</i>	8	7	6	6	6	12.372	17	15	12	10	8	0.876
<i>B. subtilis</i>	13	11	9	8	7	0.744	12	10	9	7	6	3.252
<i>E. coli</i> (Top 10)	7	6	6	6	6	>25	7	6	6	6	6	>25
<i>En. sakazakii</i>	7	6	6	6	6	>25	7	6	6	6	6	>25
<i>Shigella</i>	15	13	12	10	9	0.624	12	11	10	9	7	1.752
<i>En. faecalis</i>	7	6	6	6	6	>25	7	6	6	6	6	>25
<i>S. typhimurium</i>	7	6	6	6	6	>25	7	6	6	6	6	>25
<i>E. coli</i> (ATCC)	7	6	6	6	6	>25	8	7	7	6	6	12.372
<i>B. cereus</i>	8	7	6	6	6	12.252	7	7	6	6	6	12.252

*Lysinibacillus* sp. Gb01; *Vibrio owensii* Gb04; *Vibrio alginolyticus* Gb05; *Loktanella* sp. UKMGb03C; *Vibrio owensii* SS1; *Vibrio alginolyticus* SS17; *Bacillus subtilis*; *Escherichia coli* (Top 10); *Enterobacter sakazakii*; *Enterococcus faecalis*; *Salmonella typhimurium*; *Escherichia coli* (ATCC); *Bacillus cereus*

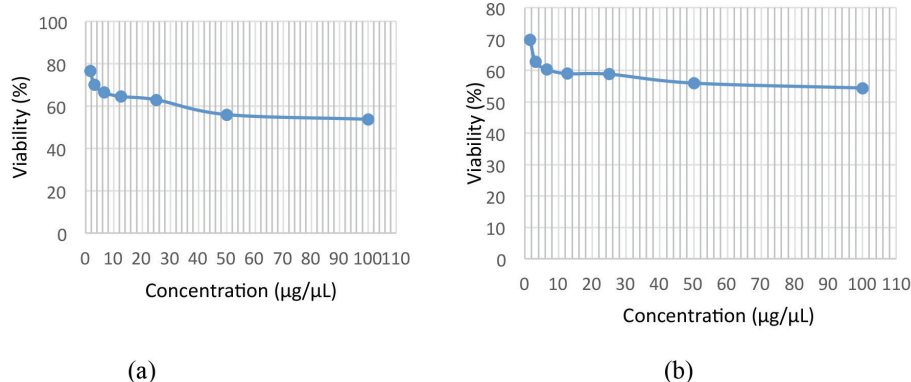


FIGURE 4. (a) Effect of **1A** on CCD841 cells (72 h exposure) (b) Effect of IPC on CCD841 cells (72 h exposure)

ranges of compounds **1A** and **1B** extracts with identified concentrations; 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µg/mL were used during the test. The test indicated that both compounds **1A** and **1B** are safe to be used as an antimicrobial therapeutic agent due to their non-toxicity towards CCD841 Normal human colon epithelial cell line with a cell viability value of 55.990% and 55.997% at a concentration of 50 mg/mL for compounds **1A** and **1B**, respectively. IC<sub>50</sub> values can be calculated from the graph of percentage of cell survival viability versus compound concentration (Figure 4). The IC<sub>50</sub> values for compound **1A** are 1.119 mg/mL and that of compound **1B** is 1.120 mg/mL. According to Zirihi et al. (2005), a test compound is considered toxic if the IC<sub>50</sub> value is less than 0.02 mg/mL. However, when IC<sub>50</sub> values were used to calculate selectivity index (SI) the antibacterial values using the equation, SI=IC<sub>50</sub>/MIC, for most of the bacteria the SI values were lower than 1, except for compound **1A** against *B. subtilis* (SI=1.50), *Shigella* (SI=1.80) and compound **1B** against *V.ovensi* SI=1.50, *V. alginolyticus* (SI=1.28). Although for both of the compounds the IC<sub>50</sub> value is high, indicating non-toxicity, but the high MIC value shows weak anti-bacterial activities.

#### CONCLUSION

Two new bis-thiourea derivatives featuring amino acid (leucine) have been successfully synthesized and fully characterized by using spectroscopic techniques. The spectroscopic results showed that both isomers exhibit no significant structural differences. However, with isomer **1B** we observed some additional peaks in the aromatic region in NMR spectrum, due to position of substituents on the central benzene ring. The dissociation constant K<sub>d2</sub> values were very low as compared to K<sub>d1</sub> which enlighten the fact that complexation was favoured at carboxylate binding site. The stoichiometry of the complex formation between host-guest for both isomers was found to be 1:4, except for isomer **1A**-Cu<sup>2+</sup> which was 1:2. UV-vis binding studies with metal cations clearly established the presence of two binding sites for both isomers. Both compounds showed non-toxic behaviour, but weak antibacterial activities.

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